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Anti-androgenic activity of hydroxyxanthones in prostate cancer LNCaP cells

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ABSTRACT

Anti-androgens are used to treat prostate cancer. Here, we report that hydroxyxanthones from a plant extract act as anti-androgens in androgen receptor (AR)-positive prostate cancer LNCaP cells. Anti-androgenic activity of the ethanol extract from *Garcinia subelliptica* was observed in a luciferase assay using LNCaP/MMTV cells with a stably integrated mouse mammary tumor virus (MMTV) promoter. HPLC-based activity profiling followed by a chemical library-based assay strategy enabled the rapid identification of several active principles bearing a xanthone core substituted with hydroxyl and isoprenyl groups. Among the active compounds, 2-(1,1-dimethyl-allyl)-1,4,5,6-tetrahydroxyxanthone (subelliptenone F) was identified as a potent inhibitor of AR transcriptional activity. The structure–activity relationship of some substituents on the xanthone core was also determined using the chemical library-based bioassay. A quantitative RT-PCR analysis revealed that treatment with the compound resulted in a significant reduction in AR-induced gene (*KLK3*) expression. Hydroxyxanthone may be a possible candidate for the development of a new anti-androgenic molecule.

1. Introduction

In men, prostate cancer is the most common cancer and the second leading cause of cancer-related death in Western countries [1]. The growth and progression of prostate cancer are initially androgen dependent. Therefore, androgen ablation therapy with luteinizing hormone-releasing hormone analogs and anti-androgens achieves significant clinical responses, and has been the standard treatment for prostate cancer [1]. Although the efficiency of androgen ablation therapy is 70–80%, prostate cancer often progresses to an androgen-independent state several years after the initial therapy, and recurrent tumors are difficult to cure. Taxane treatment is only available for the patients with androgen-independent prostate cancer. Hence, there is a need to develop novel therapeutic agents for the treatment of prostate cancer, including more potent anti-androgens.

Various novel compounds with potent biological activities have been developed from natural plants. Many researchers have performed screening experiments to find novel anti-androgens from natural products, and several compounds or extracts derived from dietary plant material have been identified [2–4]. For example, resveratrol (a stilbenoid), a phytoalexin produced by a variety of plants such as grapes and berries, was shown to inhibit androgen-stimulated cell growth and gene expression through its effect on androgen receptor (AR) signaling [2,4]. Green tea (*Camellia sinensis*) was found to have an anti-androgenic effect through 5α-reductase inhibition by epigallocatechin (a flavonoid), its major polyphenol [3]. Despite the number of polyphenol classes that exist, including flavonoids, stilbenes, coumarins, and xanthones, no comprehensive investigation on the anti-androgenic effect of xanthones has been performed.

Plants belonging to the family Clusiaceae are known to be rich in xanthone derivatives, which is a group of bioactive molecules in the polyphenols that have been identified in this family. Various biological activities of xanthones have been

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exhibited. Chemical libraries from Clusiaceae plants of the genera *Garcinia*, *Calophyllum*, *Mammea*, *Mesua*, *Harungana*, and *Poeciloneuron* were accumulated during our studies from 1994 to 1998 [5–15]. *Garcinia subelliptica* (Clusiaceae) is an evergreen tree that is distributed from Okinawa Island in Japan to Taiwan and the Philippines. The woody parts of this plant are rich in secondary metabolites of polyphenols, including xanthones, benzophenones, and biflavonoids. We previously isolated new hydroxyxanthones (subelliptenones A–I), together with known derivatives from the root bark [5,6,8,9], and bioassays using this chemical library have been conducted to identify various biological activities, such as anti-cancer and anti-bacterial activities [16,17]. As part of our search for a potent anti-androgen for the treatment of prostate cancer, we examined the anti-androgen effects of some plant-derived organic extracts and investigated the potency of the stem bark ethanol extract of *G. subelliptica*. This paper reports on the bioassay-guided fractionation and the identification of 2 effective hydroxyxanthones in the active fraction, as well as the other active derivatives identified from the chemical library and structure activity relationship (SAR) aspects.

2. Materials and methods

2.1. General experimental procedures

Semipreparative HPLC was performed on a Shimadzu HPLC system (Shimadzu Kyoto, Japan) that consisted of an SCL-10AVP system controller, 2 LC-6AD pumps, a DGU-20A3 on-line degasser, a CTO-10AVP column oven, a SIL-10AXL autosampler, and an SPD-10A UV–VIS detector. Separations were performed on a Capcell Pak C18 column (UG120, 250 × 10 mm i.d., SHISEIDO, Tokyo, Japan). The chromatographic data were collected and processed using Shimadzu CLASS-VP software (version 6.14, Shimadzu, Japan). Parallel evaporation of fractions obtained by HPLC was undertaken using the BUCHI Syncore Polyvap system (BUCHI, Flawil, Switzerland).

2.2. Biological materials

The stem bark of *G. subelliptica* (1.0 kg) was collected from the village of Kunigami in Okinawa, Japan (February 2012). The plant material was identified by one of the authors (M.I.). A voucher specimen (number GSSB_1202) was deposited with the herbarium at Gifu Pharmaceutical University.

2.3. Preparation of the extract and fractions for bioassay

A portion of the material (100 g) was dried, ground, and extracted with ethanol (1 L, 24 h) at 25 °C. The solution was then filtered. The extraction process was performed 3 times. Each filtrate was mixed and concentrated in vacuo at 40 °C by using a rotary evaporator equipped with a coolant system to produce a dry solid mass of 12.0 g (ethanol extract of *G. subelliptica* [stem bark]: E-GSSB). The extract, as well as the other screening library that consisted of plant extracts, were stored as 50 mg/mL solutions in DMSO for the primary bioassay. To localize the bioactivity of the extract, E-GSSB was separated on a C_{18} semi-preparative column. Mobile phases A and B were water and CH_3CN, respectively. The column temperature was 40 °C. A combination of isocratic and gradient mobile phase systems was used: 0–5 min, 10% B (isocratic); 5–50 min, 10–100% B (linear gradient); 50–60 min, 100% B (isocratic). The detection wavelength was 326 nm. The flow rate was 5.0 mL/min, and 50 μL volumes corresponding to 10 mg of E-GSSB were injected. Fractions were collected at 5-min intervals into vials, and each fraction (25 mL each) was evaporated in vacuo at 40 °C using a parallel evaporation system to yield dried twelve fractions (*F1–F12*: F1 [53.4 mg], *F2* [3.8 mg], *F3* [1.7 mg], *F4* [1.7 mg], *F5* [5.2 mg], *F6* [3.7 mg], *F7* [2.1 mg], *F8* [1.2 mg], *F9* [1.0 mg], *F10* [1.4 mg], *F11* [1.2 mg], and *F12* [1.5 mg]). Each fraction was dissolved in DMSO (200 μL) for the assessment of its anti-androgenic activity.

2.4. Compounds for identification and bioassay

Hydroxyxanthones previously isolated from some Clusiaceaeous plants were used for HPLC-aided identification in the active fractions and bioassay for the SAR study. The chemical structures of the compounds tested in this study are listed in Fig. 1. Subelliptenones F (**1**), C (**2**), G (**5**), E (**6**), and A (**7**) were isolated from the root bark of *G. subelliptica*, whereas 2-(1,1-dimethyl-allyl)-1,4,5-trihydroxyxanthone (**3**), 4-(1,1-dimethyl-allyl)-1,2,5-trihydroxyxanthone (**4**), and garciniaxanthones A (**8**) and B (**9**) were isolated from the bark of *G. dulcis*. The isolation procedure and spectroscopic data of all of the compounds have been previously described [5,6,8–10].

2.5. Cell culture

Human prostate carcinoma LNCaP and PC-3 cells were obtained from American Type Culture Collection (Rockville, MD, USA). LNCaP/MMTV cells were established by stable transfection of MMTV-luc vector. The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin in a humidified, 5% CO_2 atmosphere at 37 °C.

2.6. Luciferase assay

For experiments with the LNCaP/MMTV cells, the cells (1×10^5 cells/well) were incubated in a 24-well culture plate (Sumilon, Tokyo, Japan) for 1 day, and treated with each sample. After incubation for 24 h, cell lysates were prepared, and luciferase activities were measured using the PicaGene luciferase assay system (Toyo Ink, Tokyo, Japan). Firefly luciferase activity was normalized to the protein concentration of the lysate.

For experiments with the LNCaP cells, the cells were (1×10^5 cells/well) were incubated in a 24-well culture plate (Sumilon) for 1 day, and co-transfected with 0.76 μg of the androgen-responsive MMTV-luc firefly luciferase reporter plasmid and 0.04 μg of *Renilla* luciferase plasmid phRL-TK using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After incubation for 24 h, cell lysates were prepared, and luciferase activities were measured using the Dual-Luciferase Reporter assay system (Promega, Madison, WI, USA). Firefly luciferase activity was normalized to the activity of *Renilla* luciferase.

For experiments with the PC-3 cells, the cells (0.5×10^5 cells/well) were incubated in a 24-well culture plate in phenol red-free RPMI-1640 medium containing 2% FBS for